METHOD 4670

TRIAZINE HERBICIDES AS ATRAZINE IN WATER BY QUANTITATIVE IMMUNOASSAY

1.0 SCOPE AND APPLICATION

1.1 This method describes a procedure for the quantitative determination of atrazine (CAS# 1912-24-9) and other triazine herbicides in water using a competitive immunoassay. The method provides a single quantitative result, reported as atrazine, for all compounds detected. However, the extent to which other triazine herbicides and other compounds are detected may vary between commercial testing products (see Secs. 1.4 and 4.0).

1.2 Testing products are commercially available from several manufacturers. The testing product evaluated by EPA for this method employs a competitive immunoassay. Other products differ in a number of respects, including the format of the test (tubes versus microtiter plates), the reagents used, and the specific steps in the test procedure.

1.3 The method detection limit (MDL) submitted by the manufacturer of the testing product described in Sec. 6.2 was 0.03 µg/L for drinking water samples. The actual detection limit may be highly dependent on the sample matrix and analyst's performance.

1.4 Since immunoassay methods use antibody molecules that can bind to more than the target analyte, an immunoassay has a tendency to overestimate the concentration of the target analyte when other analytes are present that may bind with the antibody. The commercially-available testing product evaluated for this method is based on an immunochemical reaction that will also respond to other triazine compounds. These other triazine compounds are often included in pesticide formulations containing atrazine. Thus, the specificity of this procedure for atrazine is partly a function of the cross-reactivity of those other compounds (see Table 1). Therefore, as with other analytical techniques such as single-column gas chromatography, it is advisable to confirm positive test results near or above a regulatory action limit when the presence of other triazines is suspected.

1.5 This method is restricted to use by or under the supervision of analysts trained in the performance and interpretation of immunoassay methods. Each analyst must demonstrate the ability to generate acceptable results with this method (see Sec. 9.5).

2.0 SUMMARY OF METHOD

2.1 An accurately measured volume of sample (as little as 200 µL for some testing products) is mixed with a volume of enzyme-atrazine conjugate reagent in a test tube or a microtiter plate that has an anti-atrazine antibody immobilized on the surface, or in a vessel to which particles (magnetic particles for one testing product) with an immobilized antibody on the surface are added. The conjugate "competes" with the atrazine present in the sample for binding to the immobilized anti-atrazine antibody. The mixture is incubated at the temperature, and for the time, described in the manufacturer's instructions. (Testing products may employ other solid-phase support configurations, or even eliminate the solid-phase support. The summary here is intended to be generic and not to limit the development of other testing products).

2.2 Unbound conjugate and sample analyte that may be present in the tubes or wells are removed by washing with organic-free reagent water or wash solution specified by the manufacturer. A signal-generating substrate/chromogen reagent is added and the tube or plate is incubated as described in the manufacturer's instructions. In the case of the testing product described in Sec. 6.2, a magnetic field is applied to the tubes to retain the magnetic particle coated with antibody and any bound enzyme conjugate present during the wash step. (Other testing products may use different configurations).

2.3 In an enzyme immunoassay, a stop solution is added to the tubes or wells of the plate to terminate the signal generating activity of the enzyme conjugate reagent. The absorbance is measured at a wavelength specified by the manufacturer. The test is interpreted by measuring the signal produced by a sample and determining the concentration from a dose-response curve constructed from standards tested at the same time. For a competitive immunoassay, the color (signal) developed during the test is inversely proportional to the concentration of atrazine in the sample.

3.0 DEFINITIONS

The definitions associated with immunoassay procedures are given in Method 4000 and in the glossary at the end of this method.

4.0 INTERFERENCES

4.1 Compounds that are chemically similar may cause a positive test result (false positive) for atrazine. This phenomenon is known as cross-reactivity. The testing product used in preparation of this method has been evaluated for cross-reactivity by the manufacturer. Table 1 provides the concentration at which known cross-reactants will give a comparable response to that of atrazine when present in the sample.

4.1.1 The presence of cross-reacting compounds will result in an increase in the calculated concentration of the sample being analyzed and therefore influence the incidence of false positive results. Thus, from the standpoint of monitoring compliance with a regulatory action limit, cross-reactivity is not a significant concern for test results *below* the action limit.

4.1.2 As with techniques such as single-column gas chromatography, in instances where the presence of other triazine compounds is known or suspected, it may be advisable to confirm positive results near or above the regulatory action limit using another analytical technique. However, false negative results are generally not a concern with immunoassay techniques.

4.2 Non-specific interferences such as sample pH, temperature, osmolarity, solvents, surfactants, and the presence of metal ions can effect immunoassay performance. Samples should be tested at the pH and temperature range specified by the testing product manufacturer. Review the product literature with regard to other potential interferences.

4.3 Storage temperatures may alter the useful life of the testing product reagents and supplies. Follow the manufacturer's directions for storage and use of all reagents and supplies.

5.0 SAFETY

No extraordinary safety measures are required. However, safety procedures consistent with good laboratory practices should be employed. Some reagents may contain dilute acid solutions. Avoid contact with eyes, skin, and mucous membranes.

6.0 EQUIPMENT AND SUPPLIES

6.1 Each commercially-available testing product will supply or specify the apparatus and materials necessary for successful completion of the test. Most testing products supply the equipment and supplies specific to the immunoassay, including the tubes or plates containing the immobilized antibody, and the immunochemical reagents. Do *not* mix the equipment, supplies, and reagents from the testing products for different analytes, or from the testing products from different manufacturers. Testing products contain immunochemical reagents that are evaluated by the manufacturer on a lot-specific basis. Do not mix the reagents from one lot with those from another lot unless expressly allowed by the manufacturer. Other equipment that may be required, but is not supplied with the testing product, includes common laboratory items such as precision pipetting devices, vortex mixers, etc.

6.2 The immunoassay testing product listed below has been submitted to EPA, evaluated by the Agency, and found to meet the performance specifications necessary for inclusion in SW-846. Additional testing products may be available from other manufacturers or in different formats. As additional testing products are evaluated by EPA and found to provide equivalent performance, information will be made available by the Office of Solid Waste regarding all those testing products that are capable of meeting the performance specifications in this method. However, this procedure will not be revised solely to include information on additional testing products.

Atrazine RaPID Assay® (Ohmicron Environmental Diagnostics, Inc.).

7.0 REAGENTS AND STANDARDS

As with the equipment and supplies, each commercially-available testing product will supply or specify the reagents necessary for successful completion of the test. This includes the calibrators (standards) employed in the immunoassay. As noted in Sec. 6.1, do *not* mix the equipment, supplies, and reagents from the testing products for different analytes, or from the testing products from different manufacturers. Store all reagents and standards according to the manufacturer's instructions, and, where applicable, discarding any which have exceeded the expiration date assigned by the manufacturer.

In addition, in order to demonstrate the method performance described in Sec. 9, the following reagents and standards will be required.

7.1 Organic-free reagent water - All references to water in this method refer to organic-free reagent water, as defined in Chapter One. Organic-free reagent water is used for the preparation of the initial demonstration of capability test, the laboratory control sample, and other quality control tests. These tests are in addition to any control material(s) supplied by the manufacturer.

7.2 Atrazine spiking solution - a solution of atrazine in a water-miscible solvent is required for spiking into organic-free reagent water to prepare the initial demonstration of proficiency test, the laboratory control sample, and other quality control tests. This solution may be provided by the

manufacturer. If not provided, the laboratory should prepare a spiking solution or purchase one from a commercial source. Consult the manufacturer's instructions regarding solvents that may interfere with the testing product and do not use them. The concentration of this solution should be approximately 0.3 µg/mL, such that a 100 µL volume spiked into a 10 mL volume of reagent water will yield a concentration of 3 µg/L. Other volumes and concentrations may be employed, provided that the laboratory can demonstrate that the volume of solvent used does not affect the test performance.

7.3 Solutions for adjusting the pH of samples before extraction, where such pH adjustment is specified by the manufacturer.

7.3.1 Sulfuric acid solution (1:1 v/v), H_2SO_4 - Slowly add 50 mL of H_2SO_4 (sp. gr. 1.84) to 50 mL of organic-free reagent water.

7.3.2 Sodium hydroxide solution (2N), NaOH - Dissolve 8 g NaOH in organic-free reagent water and dilute to 100 mL.

8.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

Sample collection, preservation, and storage requirements may vary by EPA program and may be specified in the regulation that requires compliance monitoring for a given contaminant. Where such requirements are specified in the regulation, follow those requirements. In the absence of specific regulatory requirements, use the following information as guidance in determining the sample collection, preservation, and storage requirements.

8.1 Sample Collection

The immunoassay testing products employ very small (< 1 mL) sample volumes. Therefore, sample collection procedures should focus on the volume necessary to ensure that the sample represents the source.

8.1.1 Samples should be collected in pre-cleaned glass containers.

8.1.2 When sampling from a water tap, open the tap and allow the system to flush until the water temperature has stabilized (usually 2 to 5 minutes). Adjust the flow to about 500 mL/min, and collect samples from the flowing stream. When sampling from an open body of water, fill the sample container with water from a representative area.

8.2 Sample preservation

8.2.1 If residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample to the bottle, prior to collecting the sample.

8.2.2 Retard microbiological degradation by adjusting the pH of the samples to <2 with hydrochloric acid at the time of sample collection. Before analysis, readjust the pH of the samples to the pH specified by the manufacturer with 2N NaOH. The pH of the entire collected sample should be adjusted, not just the small volume utilized for the analysis.

8.3 Sample storage - Samples should be stored at 4 ± 2°C until analysis, but must be warmed to the temperature specified by the manufacturer for analysis.

8.4 EPA has not conducted holding time studies relative to immunoassay.

9.0 QUALITY CONTROL

As noted in Sec. 1.2, the specific formats of the commercially-available testing products vary by manufacturer. As a result, those testing products evaluated and accepted by EPA represent performance-based analytical methods. **Therefore, it is imperative that the manufacturer's instructions and specifications be followed closely.** Follow the manufacturer's instructions for the testing product being used for quality control procedures specific to the testing product used. The following discussion of quality control requirements relies heavily of the analyst's knowledge and understanding of the manufacturer's instructions.

9.1 Initial calibration

An initial calibration must be performed concurrent with the analysis of any samples, as described in Sec. 10.

9.2 Calibration verification

Calibration verification is not performed in the traditional sense because the initial calibration standards are analyzed with each batch of samples each time the analyses are performed.

9.3 Routine Quality Control

Routine quality control procedures associated with this method include the analyses of standards, matrix spike samples, laboratory control samples, method blanks, and duplicate or replicate analyses (as specified by the manufacturer). All of the analyses described below must be conducted simultaneously, e.g., as part of the same batch of samples. A batch of samples consists of up to 20 field samples prepared and analyzed at the same time, *or* the maximum number of samples that can be analyzed along with the standards, controls, and other analyses specified by the manufacturer using a single testing product, *whichever is fewer*. The batch must include any duplicate or replicate analyses specified by the manufacturer as well as all additional quality control tests specified by EPA in this procedure.

9.3.1 Calibration standards must be analyzed concurrently with each batch of samples processed.

9.3.2 Matrix spike (MS) samples must be analyzed with each batch of samples processed. The matrix spike samples should contain atrazine at the regulatory limit of interest (e.g., the MCL for the Drinking Water Program). The sample chosen for spiking should be representative of the field samples being analyzed.

9.3.3 The analyst must evaluate the accuracy of the assay by analyzing a laboratory control sample (LCS) consisting of organic-free reagent water sample spiked at the regulatory limit of concern for atrazine. For the Drinking Water Program, the LCS must be spiked at 3 µg/L (the MCL for atrazine) with the spiking solution in Sec. 7.2. The mean recovery (bias) of the assay must be between 80-120%. If the manufacturer does not supply the spiking solution described in Sec. 7.2, or if another regulatory limit is relevant, then the laboratory is responsible for purchasing or preparing an appropriate spiking solution and performing this test. An LCS must be prepared and analyzed with each batch of samples analyzed.

NOTE: Spiking at 3 µg/L may require that the sample be diluted to be within the calibration range for some testing products, however, it provides data regarding the bias (if any) at the regulatory threshold, as well as indications of the analyst's proficiency at making dilutions.

9.3.4 A method blank, consisting of a volume of organic-free reagent water (see 7.1) equal to that of a field sample, must be analyzed with each batch of samples processed. The method blank should not contain any detectable atrazine.

9.3.5 Samples should be analyzed in duplicate or triplicate, as instructed by the manufacturer. The number of replicate analyses is specified by the manufacturer, and is a function of the overall precision of the particular testing product. If the manufacturer determines that, in order to achieve the precision claimed by the manufacturer, a given number of replicate analyses must be performed, then the laboratory must employ the specified number of replicate analyses.

9.4 Sample Dilutions

If the sample concentration is outside of the calibrated range demonstrated by the initial calibration and as specified by the manufacturer, then the sample must be diluted to within the calibration range and re-tested. As employed in these testing products, the calibration range specified by the manufacturer is based on a B/B_0 in the 0.2-0.8 (20-80%) range. Given the nature of the competitive immunoassay, the sample cannot be diluted ***after*** color development. Thus, a diluted aliquot of the original sample must be prepared and analyzed.

NOTE: The B/B_0 range of 0.2-0.8 is *narrower* than the simple concentration range of the calibration standards. Therefore, the decision to dilute a sample for reanalysis must be based on an evaluation of the B/B_0 value of the sample, and *not* on a simple comparison of the concentration in the sample and the highest standard in the calibration.

9.5 Initial demonstration of proficiency

Each laboratory must demonstrate initial proficiency with the testing product that it utilizes, by generating data of acceptable accuracy and precision for a reference sample containing atrazine in a clean matrix. The laboratory must also repeat this demonstration whenever new staff are trained or significant changes in instrumentation are made.

9.5.1 The reference sample is prepared from a spiking solution containing the analyte of interest (see Sec. 7.2). Given the very small sample volume required for the immunoassay, a single 10-mL aliquot will provide sufficient volume for multiple tests and minimizes the difficulties involved in spiking small volumes of organic-free reagent water. Prepare a new aliquot each time the initial demonstration is to be performed.

9.5.2 Prepare an aliquot of organic-free reagent water, spiking it with the solution in Sec. 7.2 to yield a concentration of 3 µg/L. Mix the aliquot well and allow the spiked sample to stand for at least one hour.

9.5.3 Analyze at least four replicate subsamples of the spiked organic-free reagent water aliquot using the same procedures used to analyze actual samples (Sec. 11). Analyze the number of replicates of each subsample specified by the manufacturer, e.g., if the manufacturer specifies triplicate analyses of samples, then analyze 12 replicates (4 x 3) of the spiked sample.

9.5.4 Calculate the mean recovery ($\overline{X}$), and the standard deviation of the recoveries using the total number of replicate results, as described in Sec. 12.6.

9.5.5 Given the total number of replicate analyses performed, the mean recovery ($\overline{X}$) should be in the range of 90-110% and the relative standard deviation should be no more than 10% of the mean recovery. If the results fall outside of these acceptance limits, recheck all calculations. If no errors are found, repeat the demonstration until the specifications are met.

9.6 Other Quality Control Considerations

9.6.1 Do not use testing products past their expiration date.

9.6.2 Do *not* mix the equipment, supplies, and reagents from the testing products for different analytes, or from the testing products from different manufacturers.

9.6.3 Use the testing products within the storage temperature and operating temperature limits specified by the manufacturer.

10.0 CALIBRATION AND STANDARDIZATION

10.1 The analyst must perform an initial calibration. This calibration is performed concurrently with the analysis of samples.

10.1.1 The initial calibration must consist of standards (calibrators) at a minimum of three concentrations that describe the quantitation range of the assay and should preferably span the regulatory limit of interest (e.g., for drinking water, the maximum contaminant level [MCL] is 3.0 µg/L). The standards must fall within the B/B_0 range of 0.2 to 0.8. The calibrators are generally provided by the product manufacturer. Calibration curves where all the calibrators are below the regulatory limit are allowed, but will require dilution and reanalysis of samples when the sample concentration is near the regulatory limit. Calibration curves where ***all*** the calibrators are ***above*** the applicable regulatory limit may *not* be employed for compliance monitoring.

10.1.2 The testing product must also contain a "zero standard" or diluent solution that contains none of the target analytes. This solution is used to generate the B_0 value, but must not be used as one of the three standards specified in Sec. 10.1.1.

10.1.3 When the entire dose response of a competitive immunoassay testing product (the absorbance of the solution or other signal specified by the manufacturer) is plotted on the y-axis against the concentration of the calibration standard on the x-axis, the resulting calibration curve will be hyperbolic when plotted on rectilinear paper, sigmoidal when plotted on semi-log paper, and linear when a Logit-log transformation of the data is employed and plotted on rectilinear paper. In addition, since the immunoassay is competitive, the blank (zero standard) will yield the highest response, with the color development inversely proportional to the standard concentration.

A plot of either the Logit B/B_0 or the Logit of the signal (absorbance units) versus the natural log of concentration is a widely used representation of the calibration data that generally yields a linear response curve. It is the basis of most computerized data analysis

algorithms for competitive binding assays. The Logit B/B_0 is calculated according to the following formula:

$$\text{Logit } (B/B_0) = \log_e \left(\frac{\frac{B}{B_0}}{1 - \frac{B}{B_0}} \right) = \log_e \left(\frac{B}{B_0 - B} \right)$$

where:

$\log_e$ = Natural log or logarithm base e
B = Response of the standard or sample
B_0 = Response of the zero standard

When Logit B/B_0 is plotted against the natural log ($\log_e$) of concentration, the results approximate a straight line with a negative slope (see Figure 1c). The transformed calibration data can then be characterized by the slope, intercept, correlation coefficient, and standard error of the line. The following sections describe the use of the Logit-log transformation of the data to prepare a calibration curve. Manufacturer's may provide software that performs these calculations and, if provided, such software should be employed according to the manufacturer's instructions.

10.1.3.1 The commercially-available testing products may specify the analysis of standards in duplicate, or even in triplicate in some testing products. Thus, a three-point initial calibration may generate six to nine calibration points. Calculate the mean response (absorbance) at each concentration, and use this in all subsequent calculations.

10.1.3.2 Following the Logit B/B_0 and log transformations described in Sec. 10.1.3, construct a first order regression line (e.g., $y = mx + b$) using Logit B/B_0 as the dependent variable (y-axis) and the $\log_e$ concentration as the independent variable (x-axis). Since the slope of the line is negative, the regression cannot be forced through the origin, as the zero standard will yield the highest response and a value of 1.0 for B/B_0. The standards used to construct the regression line all must have B/B_0 values (prior to the Logit transformation) that fall within the 0.2-0.8 range.

The correlation coefficient of the regression (r) must be at least 0.98 in order to employ the calibration curve (manufacturers may provide more stringent linearity requirements for their testing products). If r is less than 0.98, check the expiration dates of all reagents, review the procedures to ensure that all standards were incubated for the same time specified by the manufacturer, and perform a new calibration.

10.2 By convention, the working range of an immunoassay calibration curve is defined as the range of B/B_0 from 0.2 to 0.8 (or $\%B/B_0$ from 20% to 80%). Samples may be quantitated only within the working range of the curve.

10.3 As noted in Sec. 9, a new initial calibration curve must be constructed with each batch of samples assayed.

11.0 PROCEDURE

Follow the manufacturer's instructions for the test being used. These instructions are summarized in Secs. 11.1 through 11.3, however, given the difference in test formats and reagents, the discussion is generic in nature. Where the manufacturer's instructions contradict these instructions or where these instructions do not apply to a specific testing product, **follow the manufacturer's instructions.**

11.1 Prepare the samples and standards

11.1.1 Bring samples, controls, and reagents to ambient temperature. Verify that the ambient temperature is consistent with the manufacturer's recommendations and limitations for the method. Do not attempt to perform tests outside of the temperature range specified by the manufacturer.

11.1.2 Check the pH of the samples. If necessary, adjust the pH to the range specified by the manufacturer, using 2N NaOH.

11.2 Prepare the spectrophotometer, photometer, or signal measurement equipment specified by the manufacturer.

11.3 Assay samples

11.3.1 Dispense the standards, controls, and samples into the container specified by the manufacturer. Be certain to include the replicate analyses specified by the manufacturer and the routine quality control samples specified in Sec. 9.3 (also in replicate if samples are analyzed in replicate). Determine the maximum number of standards, controls, and samples that can be analyzed simultaneously and limit the number of field samples accordingly.

11.3.2 Dispense the enzyme conjugate reagent into each container as specified by the manufacturer.

11.3.3 Dispense the antibody capture reagent (where appropriate) as specified by the manufacturer.

11.3.4 Immunoassay methods employ kinetic and chromogenic reactions that are temperature sensitive. As a result, take care to perform the assay in the temperature range recommended by the manufacturer. Failure to follow temperature recommendations can lead to anomalous test results.

NOTE: Do not attempt to process more samples simultaneously than specified by the manufacturer, as the additional processing time will lead to different incubation times for the samples and standards being tested and will produce erroneous results.

11.3.5 Wash each tube or well with washing reagents, as directed by the manufacturer.

11.3.6 Dispense the signal generating and signal terminating reagents (e.g., substrate/chromogen reagent and stop solutions) to each container in accordance with the manufacturer's instructions. Pay careful attention to the incubation times specified by the

manufacturer. Failure to follow incubation time recommendations can lead to erroneous results.

11.3.7 Interpret the test results within the time specified by the manufacturer. Follow the manufacturer's instructions for determining the sample concentration. For instance, read absorbance values (or optical density) at wavelength(s) specified by the manufacturer. Follow the manufacturer's quality control and data acceptance instructions.

12.0 DATA ANALYSIS AND CALCULATIONS

As with the specific formats of the testing products and the reagents and supplies, the specifics of the required calculations may vary by manufacturer. Some testing products may provide measuring devices such as optical density readers or spectrophotometers and may include software for performing all the necessary calculations. Other testing products may require the analyst to plot results manually, using graph paper that may or may not be provided with the testing product, and determine sample results by interpolation from a standard curve. Whichever approach is used, the laboratory records (bench notes, etc.) should clearly indicate how the results were obtained and records specific to each determination, whether in hard copy or in electronic form, should be retained by the laboratory to substantiate the results.

12.1 Follow the manufacturer's instructions regarding calculation of all testing product results. Use the calibration curve generated concurrently with the sample analyses.

12.2 Where replicate test results are generated for samples or standards, calculate the mean concentration ($\overline{C}$) as:

$$\text{mean concentration} = \overline{C} = \frac{\sum_{i=1}^{n} C_i}{n}$$

where C_i is the concentration in each replicate and n is the number of replicate analyses.

12.3 For duplicate test results, calculate the relative percent difference (RPD) according to the following equation:

$$RPD = \frac{|C_1 - C_2|}{\frac{(C_1 + C_2)}{2}} \times 100$$

where C_1 and C_2 are the concentrations of the two replicate determinations.

12.4 When the manufacturer's instructions specify the analyses of three or more replicates, calculate the standard deviation (SD) and the relative standard deviation (RSD) of the replicate results for each sample, according to the following equations:

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(C_i - \overline{C})^2}{n-1}} \qquad RSD = \frac{SD}{\overline{C}} \times 100$$

where C_i is the concentration in each replicate, $\overline{C}$ is the mean concentration, and n is the number of replicate analyses.

12.5 Accuracy is estimated from the recovery of spiked analytes from the matrix of interest. Laboratory performance in a clean matrix is estimated from the recovery of analytes in the LCS. Calculate the recovery of each spiked analyte in the matrix spike, matrix spike duplicate (if performed) and LCS according to the following formula.

$$\text{Recovery} = \%R = \frac{C_s - C_u}{C_n} \times 100$$

where:

C_s = Measured concentration of the spiked sample aliquot
C_u = Measured concentration of the unspiked sample aliquot (use 0 for the LCS)
C_n = Nominal (theoretical) concentration of the spiked sample aliquot

12.6 For the initial demonstration of proficiency (Sec. 9.6) calculate the mean recovery ($\overline{X}$), and the standard deviation of the recoveries, using the results from all replicate analyses of the four subsamples. Use the equation in Sec. 12.4 for standard deviation, substituting recovery for concentration.

13.0 METHOD PERFORMANCE

13.1 Table 1 summarizes the cross-reactivity of other triazines relative to atrazine for the testing product listed in Sec. 6.2. Other testing products may have different cross-reactivity characteristics.

13.2 Table 2 summarizes the single laboratory MDL data submitted by the manufacturer for the testing product in Sec. 6.2.

13.3 Table 3 summarizes the results of a collaborative study of the immunoassay testing product described in Sec. 6.2 conducted under the auspices of the AOAC and described in Reference 3.

13.4 Figure 1 (a-c) provides three graphical representations of the calibration of atrazine using a competitive binding immunoassay such as those described here.

14.0 POLLUTION PREVENTION

Analysis for atrazine using immunoassay conforms with EPA's pollution prevention goals. Little, if any, solvent is used and minimal waste is generated.

15.0 WASTE MANAGEMENT

Laboratory waste management procedures must be consistent with federal, state, and local regulations.

16.0 REFERENCES

1. "Principles of Competitive Protein-Binding Assays," Dell, W.O., Franchimont, P., John Wiley and Sons, New York, 1983.

2. "Immunoassay Analysis and GC/MS Confirmation for Residues of Atrazine in Water Samples from a Field Study Conducted by the State of Wisconsin," Project No. 101174, Report No. ABR-91069, CIBA-GEIGY Corporation, April 6, 1992.

3. "Determination of Atrazine in Water by a Magnetic Particle Immunoassay: Collaborative Study," Hayes, Mary C., Jourdan, Scott W., and Herzog, David P., *JAOAC*, 79(2): 530-538, 1996.

4. "Performance Characteristics of a Novel Magnetic-particle-based Enzyme-linked Immunosorbent Assay for the Quantitative Analysis of Atrazine and Related Triazines in Water Samples," Rubio, Fernando M., Itak, Jeanne M., Scutellaro, Adele M., Selisker, Michele Y., and Herzog, David P., *Food & Agricultural Immunology*, 3: 113-125, 1991.

5. "Comparison of an Enzyme Immunoassay and Gas Chromatography/Mass Spectrometry for the Detection of Atrazine in Surface Waters," Gruessner, Barry, Shambaugh, Nathaniel C., and Watzin, Mary, C., *Environmental Science and Technology*, 29: 251-254, 1995.

17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

The pages to follow contain Tables 1 through 3 and Figure 1.

TABLE 1

CROSS-REACTIVITY OF RaPID ASSAY TO RELATED COMPOUNDS

Compound	CAS #	Percent Reactivity Relative to Atrazine
Atrazine	1912-24-9	100
Ametryn	834-12-8	185
Prometryn	7287-19-6	113
Propazine	139-40-2	97
Prometon	1610-18-0	32
De-ethylated atrazine	6190-65-4	22
Simazine	122-34-9	15
Terbutryn	886-50-0	13
Terbuthylazine	5915-41-3	5
Hydroxy atrazine	2163-68-0	0.5
De-isopropylated atrazine	1007-28-9	0.3
Cyanazine	21725-46-2	<0.1

TABLE 2

METHOD DETECTION LIMIT (µg/L)

Product	n	Spike Level	Std. Dev.	MDL[1]
RaPID Assay	10	0.1	0.0105	0.03

[1] The manufacturer reported MDL results for 10 replicates but used the Student's *t* value of 3.143, for seven replicates, in performing the calculations. The value shown above was corrected to the appropriate *t* value of 2.821.

TABLE 3

SUMMARY STATISTICS OF COLLABORATIVE STUDY OF RaPID ASSAY (Source: Ref. 3)

Sample Type	Spike Conc. (µg/L)[1]	n	Mean Conc. (µg/L)	Single Analyst RSD	Overall RSD	Recovery (%)[2]
Reagent Water	0.00	14	0.02	--	--	--
Municipal Tap Water	0.00	14	0.02	--	--	--
	0.15	14	0.16	19.1	39.6	107
	1.00	13	1.13	9.09	15.6	113
	3.00	14	2.85	9.08	9.08	95
Well Water	0.00	14	0.00	--	--	--
	0.15	13	0.15	16.6	43.4	100
	1.00	14	1.05	9.53	11.5	105
	3.00	14	2.88	9.95	9.95	96
Surface Water	0.00	14	0.02	--	--	--
	0.15	14	0.17	27.7	39.0	113
	1.00	14	1.06	8.22	16.2	106
	3.00	13	3.44	11.7	19.1	114
Field-Contaminated	0.00	14	0.24	29.9	35.8	--
Sample 1	0.60	14	0.93	9.27	17.9	115
	2.00	14	2.19	9.13	13.8	98
	4.00	14	3.48	8.29	9.28	81
Field-Contaminated	0.00	14	0.47	20.1	30.8	--
Sample 2	0.80	13	1.28	10.0	19.5	101
	2.00	14	3.15	7.74	18.4	134
	4.00	14	4.03	9.52	12.5	89

[1] Data for the two field-contaminated water samples represent the amount of atrazine added to the sample and the mean concentration and RSD data represent the amount found in excess of the background field contamination.

[2] Recovery not calculated for unspiked samples.

FIGURE 1

CALIBRATION DATA FROM A COMPETITIVE IMMUNOASSAY

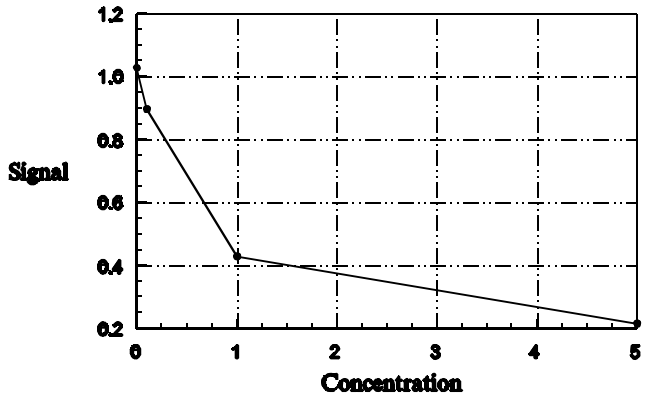

Figure 1a - Generalized plot of immunoassay signal (test response) versus concentration of calibration standard (µg/L).

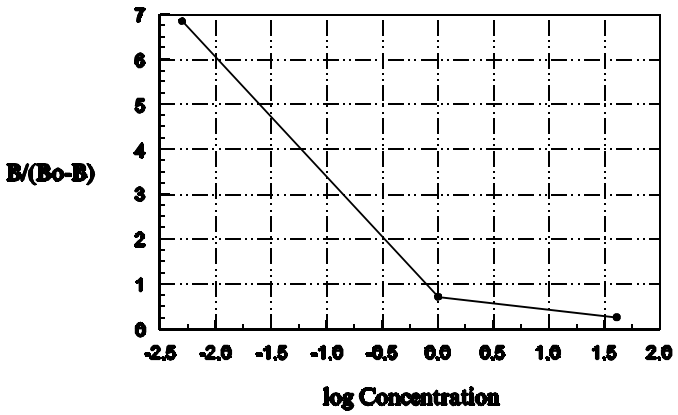

Figure 1b - Generalized plot of $B/(B_0-B)$ versus log concentration of calibration standard.

FIGURE 1
(continued)

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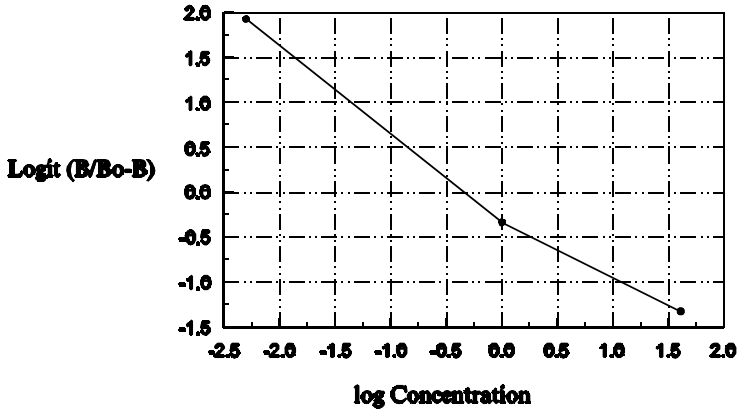

Figure 1c - Generalized plot of Logit [B/(B_0-B)] versus log concentration of calibration standard.

Antibody - A binding protein which is produced in response to an antigen, and which has the ability to bond with the antigen that stimulated its production.

$\%B/B_0$ - an indication of the displacement characteristics of the conjugate from the antibody at specified concentrations of the target compound.

$$\%\frac{B}{B_0} = \frac{\text{Response of the standard or sample}}{\text{Response of the zero standard}} \times 100$$

Competitive Immunoassay - An immunoassay method involving an *in-vitro* competitive binding reaction.

Cross-Reactivity - The relative concentration of an untargeted substance that would produce a response equivalent to a specified concentration of the targeted compound. In a quantitative immunoassay, it provides an indication of the concentration of cross-reactant that would produce a positive response. Cross-reactivity for individual compounds is often calculated as the ratio of target substance concentration to the cross-reacting substance concentration at 50% inhibition of the immunoassay's maximum signal times 100%.

Dose-Response Curve - Representation of the signal generated by an immunoassay (y axis) plotted against the concentration of the target compound (x axis) in a series of standards of known concentration. When plotting a competitive immunoassay in a rectilinear format, the dose-response will have a hyperbolic character. When the log of concentration is used, the plot assumes a sigmoidal shape, and when the log of signal is plotted against the Logit transformation of concentration, a straight line plot is produced.

ELISA - *Enzyme Linked Immunosorbent Assay* is an enzyme immunoassay method that uses an immobilized reagent (*e.g.*,antibody adsorbed to a plastic tube), to facilitate the separation of targeted analytes (antibody-bound components) from non-target substances (free reaction components) using a washing step, and an enzyme conjugate to generate the signal used for the interpretation of results.

Enzyme Conjugate - A molecule produced by the coupling of an enzyme molecule to an immunoassay component that is responsible for acting upon a substrate to produce a detectable signal.

Enzyme Immunoassay - An immunoassay method that uses an enzyme conjugate reagent to generate the signal used for interpretation of results. The enzyme mediated response may take the form of a chromogenic, fluorogenic, chemiluminescent or potentiometric reaction. (*see Immunoassay and ELISA*)

False Negatives - A negative interpretation of the method containing the target analytes at or above the detection level. Ideally, an immunoassay test product should produce no false negatives. The false negative rate can be estimated by analyzing split samples using both the test product and a reference method.

False Positives - A positive interpretation for a sample is defined as a positive response for a sample that contains analytes below the action level.

Immunoassay - An analytical technique that uses an antibody molecule as a binding agent in the detection and quantitation of substances in a sample. (*see Enzyme Immunoassay and ELISA*)

Immunogen - A substance having a minimum size and complexity, and that is sufficiently foreign to a genetically competent host to stimulate an immune response.

Logit - A logarithmic transformation of data normalized to the highest observed response. For the competitive immunoassay described in this procedure, the Logit transformation is calculated as:

$$\text{Logit}\ (B/B_0) = \log_e \left(\frac{\frac{B}{B_0}}{1 - \frac{B}{B_0}} \right) = \log_e \left(\frac{B}{B_0 - B} \right)$$

Natural Log - The logarithm, base e, of a number. The natural logarithm may also be represented as "ln" or "$\log_e$."